

ESM-1 Is a Novel Human Endothelial Cell-specific Molecule Expressed in Lung and Regulated by Cytokines*

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Philippe Lassalle†§, Sophie Molet†, Anne Janin†, José Van der Heyden||, Jan Tavernier||, Walter Fiers**, René Devos||, and André-Bernard Tonnel†

From †INSERM U416, Institut Pasteur, 1 boulevard A. Calmette, 59000 Lille, France; ‡Laboratoire d'anatomie pathologique, Hôpital Saint Louis, 1 avenue C. Vellefaux, 75010 Paris, France; ||Roche Research Gent, 22 Josef Plateaustraat, B-9000 Ghent, Belgium; **Laboratory of Molecular Biology, University of Ghent, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium

We here report the identification of a novel human endothelial cell-specific molecule (called ESM-1) cloned from a human umbilical vein endothelial cell (HUVEC) cDNA library. Constitutive ESM-1 gene expression (as demonstrated by Northern blot and reverse transcription-polymerase chain reaction analysis) was found in HUVECs but not in the other human cell lines tested. The cDNA sequence contains an open reading frame of 552 nucleotides and a 1398-nucleotide 3'-untranslated region including several domains involved in mRNA instability and five putative polyadenylation consensus sequences. The deduced 184-amino acid sequence defines a cysteine-rich protein with a functional NH₂-terminal hydrophobic signal sequence. Searches in several data bases confirmed the unique identity of this sequence. A rabbit immune serum raised against the 14-kDa COOH-terminal peptide of ESM-1 immunoprecipitated a 20-kDa protein only in ESM-1-transfected COS cells. Immunoblotting and immunoprecipitation of HUVEC lysates revealed a specific 20-kDa band corresponding to ESM-1. In addition, constitutive ESM-1 gene expression was shown to be tissue-restricted to the human lung. Southern blot analysis suggests that a single gene encodes ESM-1. A time-dependent up-regulation of ESM-1 mRNA was seen after addition of tumor necrosis factor α (TNF α) or interleukin (IL)-1 β but not with IL-4 or interferon γ (IFN γ) alone. In addition, when IFN γ was combined with TNF α , IFN γ inhibited the TNF α -induced increase of ESM-1 mRNA level. These data suggest that ESM-1 may have potent implications in the areas of vascular cell biology and human lung physiology.

the control of cytokines; E-selectin, vascular cell adhesion molecule-1, IL-8, and RANTES¹ are expressed on endothelial cells only activated by cytokines, whereas ICAM-1, ICAM-2, and IL-6, which are expressed constitutively in a low rate, are highly induced on endothelial cells in the presence of cytokines (2–8).

Vascular endothelium shows diversity among tissues, and there are fewer known mechanisms that regulate leukocyte migration and localization within specific tissues. E-selectin, GlyCAM-1, CD34, and MadCAM-1 contribute to the tissue-specific homing of circulating T lymphocytes in skin, lymph nodes, and Peyer patches, respectively. GlyCAM-1, CD34, and MadCAM-1 are mucin-like carriers of selectin ligands (9–11). CD34 and MadCAM-1 are type 1 membrane glycoproteins, but GlyCAM-1 is secreted by the high endothelial venules (9). In the other tissues, very little is known about the presence of such homing molecules on the endothelial cells. Therefore, identification of tissue- and endothelial cell-restricted molecules may contribute to a better understanding of these tissue-specific leukocyte-endothelial cell interactions.

Here we report the cloning and expression of a novel molecule specifically expressed in human endothelial cells called endothelial cell-specific molecule 1 (ESM-1). Expression of ESM-1 appears also restricted to the human lung tissue and ESM-1 can be regulated by TNF α , IL-1 β and IFN γ . To our knowledge, ESM-1 represents the first endothelial cell- and lung-restricted molecule in humans and may have potent implications in lung-specific endothelial cell-leukocyte interactions.

MATERIALS AND METHODS

Cell Culture—Human endothelial cells were derived from umbilical vein by collagenase treatment and cultured in RPMI 1640 supplemented with 20% fetal calf serum, 2 mM L-glutamine, 10 μ g/ml heparin and 25 μ g/ml endothelial cell growth supplement (Sigma). HUVECs (passage 2–3) were cultured in 0.5% gelatin-coated six-well culture plates (Falcon). At confluence, HUVECs were washed two times and incubated overnight before cytokine stimulation with RPMI 1640 containing 20% fetal calf serum. Before addition of cytokines, HUVECs were washed once and incubated in RPMI 1640 containing 5% fetal calf serum. Cytokines were then added to cell cultures of TNF α (200 units/ml, a gift from Dr. J. Wietzerbin), IFN γ (1000 units/ml, Genzyme Corp.), IL-1 β (10 units/ml, Genzyme), IL-4 (1000 units/ml, Roche Research Gent). After cytokine activation, the cells were lysed in 4 M guanidine thiocyanate buffer, and total RNA was isolated as described below.

Positioned at the interface between circulating cells and tissues, the endothelial cells play a critical role in the homing and the local accumulation of leukocytes. Initial tethering and rolling, subsequent arrest and adhesion, and transendothelial migration constitute the current view of leukocyte migration (1). Leukocyte migration involves signal molecules, including selectins, chemoattractants, and integrins, which are present on endothelial cells. Their display of signals is carefully under

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) X89426.

§ To whom correspondence should be addressed. Philippe Lassalle is a postdoctoral fellow supported by European Economic Community contract ERBCHICT941282 at Roche Research Gent. Current address: INSERM U416, Institut Pasteur, 1 boulevard A. Calmette, 59000 Lille, France.

¹ The abbreviations used are: RANTES, regulated on activation normal T cell expressed; ESM, endothelial cell-specific molecule; ICAM, intercellular adhesion molecule; HUVEC, human umbilical vein endothelial cell; IL, interleukin; UTR, untranslated region; TNF, tumor necrosis factor; IFN, interferon; bp, base pair; kb, kilobase; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

SV40-transfected human endothelial cells (SV1 cells) were cultured in RPMI 1640 containing 2 mM L-glutamine and 10% fetal calf serum (12). Dami cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% horse serum; HeLa and COS cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum; MO7 cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum; and HL60, Jurkat, Daudi, and TF1 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum.

Construction of cDNA Libraries.—Poly(A) RNA was isolated from SV1 cells using the FastTrack mRNA isolation kit (Invitrogen). Poly(A) RNA was primed with an oligo(dT)-XhoI primer adaptor (Stratagene, Inc.). cDNA was synthesized from 3 µg of poly(A) RNA as recommended by the manufacturer (Stratagene). EcoRI adaptor was added to the cDNA by blunt-end ligation following by exposure to XhoI restriction endonuclease. cDNA was fractionated by agarose gel electrophoresis and cDNA longer than 500 bp was ligated into the predigested EcoRI-XhoI sites of a Zap II vector (Stratagene). A second cDNA library was also constructed. Poly(A) RNA was primed with an oligo(dT)-NotI primer-adaptor (Promega). The buffers and enzymes used for cDNA synthesis were purchased from Life Technologies, Inc. BstXI adaptor was ligated to the cDNA blunt ends, and the cDNA was fractionated by agarose gel electrophoresis. The cDNA between 1 and 3 kb was ligated into BstXI cut pCDM8 vector. The construct was transfected into *Escherichia coli* MC1061 P3 by electroporation (Bio-Rad Gene Pulser). The libraries consisted of 1.7×10^7 plaque-forming units/µg and 10^6 independent colonies, respectively, with an average size of 2 kb.

Cloning and Sequence Analysis.—The initial 1.2-kb fragment of ESM-1 was cloned by immunoscreening of 5×10^6 recombinant phages with a rabbit immune serum raised against an immunoprecipitate of supernatant from 4×10^9 human blood platelets (13). Immunoscreening was performed as described (14). Anti-*E. coli* antibodies were removed from serum by adsorption against nitrocellulose filters pre-soaked with *E. coli* lysates. The nitrocellulose filters were incubated in a quenching buffer (5% nonfat milk in PBS) for 30 min, followed by an overnight incubation at 4 °C with constant agitation in 1:200 diluted rabbit antiserum. After washing four times with PBS, bound antibody was detected by a 2-h incubation with peroxidase-conjugated goat anti-rabbit IgG (Tago Immunologicals, Burlingame, CA). The nitrocellulose filters were then re-washed and developed with HRP color development reagent (Bio-Rad). Three distinct cDNA clones were further isolated by two rounds of purification. The recombinant pBluescript plasmids were excised *in vivo* from the lambda phage as recommended by the manufacturer (Stratagene, La Jolla, CA). These cDNAs were subsequently sequenced (T7 Sequencing Kit, Pharmacia Biotech, Inc.) and used as probes in Northern blot analysis. The full length cDNA from ESM-1 was re-cloned as following: a total of 10,000 independent colonies from a HUVEC cDNA library constructed in pCDM8 vector were lifted on nylon membranes (Hybond membranes, Amersham Corp.). Cell lysis, neutralization, washing, and UV cross-linking were performed as described (14). High stringency hybridization was performed at 65 °C in $6 \times$ SSC, $5 \times$ Denhardt's solution, 0.5% SDS, 40 µg/ml denatured salmon sperm DNA, and 0.25 mM EDTA. The filters were washed two times for 30 min at room temperature with $2 \times$ SSC, 0.1% SDS and two times for 30 min at 65 °C with $0.1 \times$ SSC, 0.1% SDS. Positive colonies were picked and purified. A cDNA of 2 kb was isolated and subsequently subjected to sequencing on both strands. Sequence analysis was performed using PCGENE (IntelliGenetics), WORDSEARCH, and FASTA (UWCGC) programs with GenBank™, EMBL, and SwissProt data bases.

Northern and Southern Blot Analysis and Probe Labeling.—Total RNA from various cells and from HUVECs was isolated by phenol and chloroform extraction followed by precipitation with isopropanol alcohol. Ten to thirty micrograms total RNA per lane were electrophoresed through 1% formaldehyde agarose gels and transferred to a nylon membrane (GeneScreen, DuPont NEN). Five micrograms of 0.24–9.5-kb RNA ladder (Life Technologies, Inc.) were used for RNA sizing. High stringency hybridization was performed at 42 °C in 1 M NaCl, 50% formamide, $1 \times$ Denhardt's solution, 10% dextran sulfate, and 100 µg/ml denatured salmon sperm DNA. The membranes were washed two times for 30 min at room temperature with $2 \times$ SSC, 0.1% SDS, and two times for 30 min at 55 °C with $0.1 \times$ SSC, 0.1% SDS. Southern blot was hybridized at 65 °C in $6 \times$ SSC, $5 \times$ Denhardt's solution, 0.5% SDS, 40 µg/ml denatured salmon sperm DNA, and 0.25 mM EDTA. The washing step consisted of two times for 30 min at room temperature with $2 \times$ SSC, 0.1% SDS and two times for 30 min at 65 °C with $2 \times$ SSC, 0.1% SDS. Hybridized membranes were exposed to x-ray film at –70 °C overnight for 2 weeks. The ESM-1 cDNA probes were

either the 1.2-kb EcoRI-XhoI initial fragment (3' end), or the 1176-bp HindIII fragment from the 5' end of the ESM-1 cDNA. The other DNA probes were: E-selectin probe (PstI-EcoRI fragment, British Biotechnology), ICAM-1 probe (EcoRI-XhoI fragment, British Biotechnology), vascular cell adhesion molecule-1 (EcoRI-XhoI fragment, a RT-PCR construction from TNF-activated HUVECs cloned in pcDNA3 (Invitrogen)) and human β -actin (Clontech Laboratories, Inc.). All of these probes were labeled by random priming (Multiprime DNA labeling system, Amersham Corp.).

Reverse Transcription.—PCR was performed with RNA from each of the cell samples shown in multiple cell Northern blot. Five micrograms of total RNA were reverse transcribed with a dT₁₂₋₁₈ oligonucleotide primer as recommended by the manufacturer (reverse transcriptase RNase H-minus, Life Technologies, Inc.). The specific primers used were ESM-1 forward primer (5'-GTTCTTTGACTTTGATGT3') and ESM-1 reverse primer (5'-CAACTCTGT-TGGCCAAACT3'); these primers yield a specific DNA fragment of 1042 bp. The human glyceraldehyde-3-phosphate dehydrogenase primers were purchased from Clontech. PCR amplification was performed for 30 cycles with a "hot start" at 92 °C for 2 min followed by 94 °C denaturation for 30 s, 51 °C hybridization for 1 min, and 72 °C extension for 1 min 30 s. RT-PCR products were resolved in a 1% agarose gel in $1 \times$ TAE buffer.

In Vitro Translation Assay.—The 5' HindIII-XbaI fragment of ESM-1 (686 bp) was cloned into pcDNA3 vector. *In vitro* coupled transcription/translation assay was performed using T7 RNA polymerase coupled with a rabbit reticulocyte lysate as described by manufacturer (Promega). One microgram of DNA was added in 25 µl of final reaction buffer containing [³⁵S]methionine. The cotranslational and initial post-translational processing of ESM-1 protein was studied by addition of microsomal vesicles, which were added at various concentrations (Promega). The translation products were analyzed by SDS-PAGE.

Fusion Protein Construction and Expression.—The XmaI-digested fragment of ESM-1 was fused in frame to glutathione S-transferase open reading frame and was expressed in *E. coli* with the use of a pGEX-2TK vector (Pharmacia). Synthesis of the fusion protein was induced by addition of 0.5 mM isopropyl-1-thio- β -D-galactopyranoside (Boehringer Mannheim) for 4 h at 37 °C. The cells were centrifuged and resuspended in PBS containing 1% Triton X-100 and a mixture of protease inhibitors (Life Technologies, Inc.). The fusion protein was purified on glutathione-Sepharose 4B column as recommended by the manufacturer (Pharmacia). After the washing step, the Sepharose beads were resuspended in 20 ml of PBS containing 10 IU of thrombin (Sigma) and incubated 2 h at room temperature. After centrifugation, the supernatants were concentrated by centrifugation on a Centricon 10 filter (Amicon), and the protein content was evaluated with a standard protein assay (Bio-Rad). One liter of bacterial culture yielded approximately 40 µg of purified ESM-1 protein.

Preparation of Polyclonal Immune Serum.—Fifty micrograms of purified ESM-1 protein were mixed with complete Freund's adjuvant and injected in a New Zealand White rabbit. Boost immunizations of 40 µg were given at 4-week intervals in incomplete Freund adjuvant. The rabbit was bled 4 weeks after the first boost immunization. Immune serum was analyzed first by immunoblotting of Sf9 cell supernatants infected with ESM-1 recombinant baculovirus. The Sf9 cells infected or not infected with the hIL-5 recombinant baculovirus (15) served as control supernatants.

Metabolic Radiolabeling and Immunoprecipitation.—The expression construct pcDNA3-ESM-1 was generated by subcloning the HindIII-XbaI insert of ESM-1 into the HindIII-XbaI-digested pcDNA3 vector. This construct was transfected into COS cells using the DEAE-dextran method. After 48 h, cells were replated into 25-cm² culture flasks, and 24 h later, both ESM-1 and mock transfectants were metabolically labeled with [³⁵S]methionine (100 µCi/ml) for 6 h. COS cell supernatants were collected by centrifugation, and COS cells attached to the plates were washed twice with PBS before cell lysis with PBS containing 0.5% Nonidet P-40 and a mixture of protease inhibitors. Five hundred microliters of PBS containing protease inhibitors were added to 500-µl COS cell supernatants and COS cell lysates. These preparations were incubated with 5 µl of rabbit immune serum and incubated overnight at 4 °C with constant agitation. Protein A-Sepharose CL-4B was added to the mixtures, which were then incubated for 90 min at room temperature. The beads were then washed by centrifugation twice with PBS, 0.5% Nonidet P-40 and twice with PBS. Twenty microliters of SDS-PAGE sample buffer were added to the beads, which were then heated at 100 °C for 2 min. After centrifugation, the supernatants were run on a 15% SDS-polyacrylamide gel. The gels were dried and exposed to x-ray film at –70 °C.

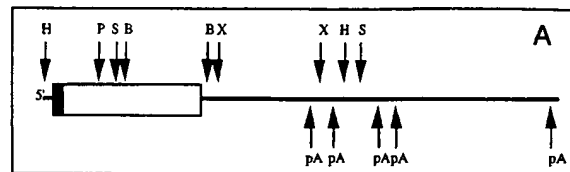
In Situ Hybridization.—Cryostat sections were fixed in 4% paraform-

aldehyde in PBS, dehydrated through graded alcohols, and stored at 4 °C. A *HindIII*-*Bam*HI ESM-1 cDNA fragment (0.3 kb from the 5' region of ESM-1) was subcloned into a pBluescript vector and linearized with *HindIII* or *Bam*HI to produce antisense or sense probes. Transcription was performed in the presence of ³⁵S-cytidine triphosphate and the appropriate T3 or T7 RNA polymerases for antisense and sense riboprobes. Hybridization and autoradiography were kindly performed by Dr. A. Tsiopoulos as described previously (16).

RESULTS

cDNA Cloning of ESM-1—The initial immunoscreening of a HUVEC cDNA library was designated to isolate cDNA encoding a 55-kDa autoantigen putatively involved in severe asthma (13). This antigen was enriched by immunoprecipitation from thrombin-activated human blood platelets, which share immunologically the common 55-kDa antigen. A rabbit antiserum was raised against this antigen and used to screen the HUVEC cDNA library. 5 × 10⁵ clones were screened with the subsequent isolation of 3 cDNA clones. These cDNA were sequenced, and sequence comparison to GenBank™ and EMBL data bases revealed unique identities for two of these three cDNAs. Multiple human cell Northern blot analysis using these cDNAs as probes revealed that one hybridized only in HUVECs and SV1 cells but not in the other cell lines tested (see below). These data suggested that this cDNA of 1.2 kb was derived from an RNA messenger that encodes a molecule highly restricted to human endothelial cells. We have called this molecule ESM-1 (endothelial cell-specific molecule 1). Thus, the full length ESM-1 cDNA was cloned in a size-selected HUVEC cDNA library constructed in pCDM8. 10,000 independent colonies were screened, and one clone was purified. This clone had shown an inverted cDNA insert of 2 kb.

Sequence Analysis of ESM-1—The complete cDNA sequence, 2006 base pairs in length, contains an open reading frame of 552 nucleotides (Fig. 1). A Kozak consensus sequence for translation initiation was identified at nucleotide 56 (AACATGA) (17, 18). The 1398-nucleotide 3'-untranslated region encodes a mRNA with 66% AU nucleotides. It includes several domains involved in mRNA instability: 10 AUUUA domains and 3 UAUUUUA(U/A) domains (19, 20). The 3'-untranslated region contains five putative polyadenylation consensus sequences (AAUAAA) (21) at nucleotides 1045, 1132, 1404, 1495, and 1987; the last one is located just 14 bp upstream from the poly(A) sequence. DNA data base searches did not reveal any significant homology with our nucleotide sequence. The cDNA is predicted to code for a protein of 184 amino acids. The predicted NH₂-terminal hydrophobic signal sequence (22) is composed of 19 amino acids. The mature protein, predicted to contain 165 amino acids, does not contain any potential N-glycosylation site. To confirm the correct assignment of the open reading frame, we performed an *in vitro* translation. ESM-1 cDNA was cloned into the *HindIII*-*Xba*I sites of the pcDNA3 vector. SDS-PAGE analysis of the primary *in vitro* coupled transcription-translation product migrated at an apparent molecular mass of 22 kDa in reducing condition. This primary translation product was shown to be truncated (2 kDa) with the addition of microsomal vesicles, which indicates that the signal sequence is functional (data not shown). ESM-1 is a cysteine-rich protein, containing 18 Cys residues. No known protein motifs were found in the ESM-1 protein. Searches with the FASTA program did not result in the identification of protein homology up to 35%. The best protein similarities were found with the human β2 integrin (cysteine-rich tandem repeats: 27.6% identity and 54.6% similarity), the human IGF binding proteins (32% identity and 55.3% similarity), the human fibrillin 1 (28.8% identity and 54.3% similarity), laminin β2 (31.4% identity and 52.6% similarity), and human procollagen α2 (24.2% identity and 44.6% similarity) (Fig. 2).



B	
CTCCACCAGCAAGACACGACTGGAGAGCCGAGCCGAGGAGCTGGGAAC	55
ATGAAGAGCGTCTTCTGCTGACACGCTCTCTGTCGTCACACCTG	103
<u>M K S V L L L T T L L V P A H L</u>	16
GTGGCCGCTGGAGCAATAATTATCGGCTGGACTGCCCTCAACACTGTGAC	154
<u>V A A W S N N Y A V D C P Q H C D</u>	33
AGCAGTCTGAGTCAGAAAGCAGCCCGCTGCAAGAGGACAGCTCGACGAC	205
<u>S S E C K S S P R C R T V L D D</u>	50
TGTGGCTGCTGGAGTGTGCGCTGACGGGCGGGGAGAACTTGCTACCGC	256
<u>C G C C R V C A A G R G E T C Y R</u>	67
ACAGTCTCAGGCATGGATGGCATGAAGTGTGGCCGGGGCTGAGGTGTGAC	307
<u>T Y S G M D G M K C G P G L R C Q</u>	84
CCTTCTAATGGGAGGATCCCTTTGGTGAAGAGTTTGGTATCTGCAAGAC	358
<u>P S N G E D P F G E E F G I C K D</u>	101
TGTCCCTACGGCACCTTCGGGATGGATTGACAGAGACCTGCAACTGCCAG	409
<u>C P Y G T F G M D C R E T C N C Q</u>	118
TCAGGCATCTGTGACAGGGGAGCGGAAATGCCGTGAATTCCTCTTCTC	460
<u>S G I C D R G T G K C L K F P F F</u>	135
CAATATTCAGTAACCAAGTCTTCCACAGATTGTCTCTCACGGAGCAT	511
<u>Q Y S V T K S S N R F V S L T E H</u>	152
GACATGGCATCTGGAGATGGCAATAATTGTGAGAGAAAGTTGTGAAAGAG	562
<u>D M A S G D G N I V R E E V K E</u>	169
AATGCTCCCGGCTCTCCCGTAATGAGGAAATGGTTAAATCCACGCTGATCC	613
<u>N A A G S P V M R K W L N P R</u>	184
CGCGTGTGATTCTGAGAGAGGCTCTATTCTGTGATTGTTCACACAGCCCACTTT	674
TAGGAATCTCTAGATATAGCATAGTACATGTAATTTTGAAGATCCAAATGTATGCA	735
TGTGGATCCAGAAACAAAGTAGGATCTTCAATCCATCACTCAATGATGCAAC	796
ACTGTGTGTGTGTATAAATGTGAATGCACTGATTTGTAAATGTGTGTATAGT	857
AACACTGAGAACTAAATGCAATTTAGTAACTTACATGGAGACAGGCTCAACAAAG	918
GGAGCTAGCAAGCTGAAGACCGCAGTGAATCAATTTAGTCTTTGACTTTGATGACA	979
TAAATGTGTGGATATGGATGAGACTTAAAGCAGAGAGAGTGGAGGGGTGGAGT	1040
GGGAATAAATAATTTAGCCCTTCCTGTAGTGTAGCTTCTAGAAATTAATGTGCTT	1100
TTTTTTTTTTTTCGCTTGGGAAAGTCAAAATTAACCAACAGAAACCCCTGAAGA	1160
AGTAGATGTGGAAGCTTATGGAAATTTGAGTACCAACAGCTTTGACTGAGAGCAAT	1221
TCAGAGGCTCTGATGTAGTTTCCCGGTTTACCTGTATCTGAGGAGCGGTTCTGGGCA	1282
GGAAACACATACCTTCCATTAATAGCTTTAGCTATGTCGACCTCAGAGATTAATCTAGA	1343
AGTATTTTCCCACTGGTGTGTGTGTGTATGAGGTAAATTTATATATTTTATAA	1404
ATTAATGTGTATAGTCAAGTCACTTCCCTACCACTATTTATCTATCTCTTGAAGAA	1464
AACTAGTATTTATTTGTGAAATGGTTGAAGAAACCTATGACTCTATAAGGTTTTC	1524
AAACATCTGAGGATGATTAATTTTATCCATTAATTAAGGACTCACTCTGGATTTCAA	1585
AAATCTCAAAATGAGCAACAGAGGACCTTATTTTAACTAATGCTGTGACTTCGGTG	1646
AAATTTCAATTTAGGTATGAATAAGTTTATAGAGGTTTGTAAAGAGAAATCAATTT	1707
TCAGCAAGAAACATGTCACTTTAAATATAGGTGGAATTTAGGAGTATTTTGAAGATC	1768
TTAGCAAGAAAGGACTGTGTATGATGAGTTCTTGAAGATTTATCTCAGAGTATTTTAT	1829
TGAAGTGAAGAACTTATTTAGAAATTTTCAATTTTACCTGTATTTTATCTGAAGTT	1890
GGCCACAGAGTTGTGATGTGTGTGAGGCTTTGATGTAAAGCTCATAGCTGTTA	1951
GGTTTGTGTAAAGGACATGTTTATTTATTTCTCAATTAAGAAAGACAGATC 3'	2006

Fig. 1. Restriction map, nucleotide sequence of ESM-1 cDNA, and predicted protein structure of ESM-1. A, partial restriction map of ESM-1 cDNA. H, *HindIII*; P, *PstI*; S, *SmaI*; B, *Bam*HI; X, *XbaI*; pA, polyadenylation site. The putative signal peptide is the *dark box*. The coding sequence is in *white box*. B, nucleotide and predicted amino acid sequence of ESM-1. Double underline indicates putative signal sequence. The mRNA decay consensus sequence motifs are underlined and potential polyadenylation sites are in *bold type*.

ESM-1 Gene Expression—Multiple human cell lines were analyzed by Northern blot. ESM-1-specific DNA probes hybridized strongly to HUVECs and SV40-transfected HUVECs (Fig. 3A). One major band was seen with an apparent size of 2.2 kb. A weak band was also seen in COS cells, but this band was considered nonspecific because its size corresponded to the 28S rRNA, and RT-PCR analysis with COS RNA did not reveal any specific band compared to HUVEC RNA (Fig. 3B). No other bands could be detected, whatever probe was used (probes included either the coding sequence or the 3'-UTR). Strikingly, this band was undetectable in any of the cell types other than HUVECs and SV1 cells, even after x-ray exposure up to 2 weeks. Apparent cell-specific expression was also observed with RT-PCR of RNA from each of the cell samples using specific primers from ESM-1 cDNA. A strong signal of 1042 bp was seen only in HUVECs, and

ESM-1	19	AWSNNYAVDCPQH	SSSECK--SSPR	---XRTVLDD
itb2	523	DVFMKKIYGQFCR	CDTINERYWGV	CGGPGRLCLP
ESM-1	52	CGRCRVA--A	ROETCYRYVS	--MDGKXGFG
itb2	559	CGRCRA--H	PGFRRSACQ	EBRTYRCLMPRRVZ
ESM-1	81	LRCPSPNGDPT	EEFGIKDCPY	GTFCHDCRETEN
itb2	594	GRRCRCNVCE	CHSUYOLPL	OEKRCGCFSPCGEYIS
ESM-1	116	CQSGICDRGT	CLKPPFP	FOYSVTSSNRVSLT
itb2	629	ECLKPEKGF	NCSSAAC	PGQLQSNPNPVKGRCTCR
ESM-1	152	HAAGDGNIVR	EVVKEAAGSP	MRKWLNPR
itb2	665	DSREGCMVAYTL	EQDDGMORYLIY	DESRECVAGPN
ESM-1	19	AWNNYAVDCPQH	SSSECK--SSPR	---XRTVLDD
itb2	51	ICLDIRLETF	FLRYEDF	CTL--IAGRRMRMA
ESM-1	54	CGRCRVA--A	ROETCYRYVS	--MDGKXGFG
itb2	559	CGRCRA--H	PGFRRSACQ	EBRTYRCLMPRRVZ
ESM-1	81	LRCPSPNGDPT	EEFGIKDCPY	GTFCHDCRETEN
itb2	106	TRKITNCR	PKFDINE	CHMISLCTHOKR
ESM-1	116	CQSGICDRGT	CLKPPFP	FOYSVTSSNRVSLT
itb2	154	XCRCL	SGFALDSERNCT	DDICRISPOLCGRGQCV
ESM-1	152	HAAGDGNIVR	EVVKEAAGSP	MRKWLNPR
itb2	190	HTPCDGF	CKCDEGY	SGFAAAXNCMDIDECQRD
ESM-1	19	AWNNYAVDCPQH	SSSECK--SSPR	---XRTVLDD
itb2	58	PCQICVCDNGAIL	CKKIE	QDDVLD
ESM-1	55	RVCACAGAG	ETCYRTYS	MDGKXGFG
itb2	93	PCVCSOTF	GGHTFGR	ARKGO
ESM-1	91	PFPG	EEFGICKDC	PYCTFMD
itb2	130	RFPG	PAGFP	SGGQGRGER
ESM-1	127	CKLCKPFP	FOYSVTSSNRVSLT	EDHNASGDCNIV
itb2	165	VPGQ	PGADGPP	CHPSHP
ESM-1	163	REEVKNEAAGSP	MRKWLNPR	
itb2	201	SOLCSQVGLH	FGS	VG
ESM-1	19	AWNNYAVDCPQH	SSSECK--SSPR	---XRTVLDD
itb2	775	ASGLVYNGAL	CCQCP	PGGSL
ESM-1	56	RVCACAGAG	ETCYRTYS	MDGKXGFG
itb2	809	PGVVGRC	CDATG	TYF
ESM-1	91	PFPG	EEFGICKDC	PYCTFMD
itb2	843	ALC	EGTSG	Q
ESM-1	126	TKCLKPPFP	FOYSVTSSNRVSLT	EDHNASGDCNIV
itb2	876	RCV	CN	GRAD
ESM-1	162	REEVKNEAAGSP	MRKWLNPR	
itb2	912	GFHGD	RL	FL

FIG. 2. Comparisons of the sequence of ESM-1 to amino acid sequences of several proteins. Shown is alignment of sequences computerized by the FASTA program. The ESM-1 sequence is shown in the first line and compared with integrin $\beta 2$ (*itb2*), fibrillin 1 (*fbn1*), procollagen $\alpha 2$ (*pca2*), and laminin $\beta 2$ (*lmb2*). Amino acid identity is boxed.

glyceraldehyde-3-phosphate dehydrogenase was detectable in all cell lines tested (Fig. 3B). Hybridization of poly(A) RNA from various human tissues revealed a constitutive expression of ESM-1 in the lung (Fig. 4), whereas lower levels were detected in kidney. The major band detected was found to be similar in size to that in HUVECs (2.2 kb). In addition, no ESM-1 mRNA was detectable in the heart, pancreas, placenta, brain, striated muscle, or liver, even after a 2-week exposure. To determine the endothelial cell origin of ESM-1 in the human lung, we performed *in situ* hybridization on frozen sections of a normal human lung tissue. Hybridization with an 35 S-labeled antisense probe revealed a strong signal exclusively localized on the vascular capillary network within alveolar walls (not shown). Capillary veinules and arteriolar were both positive. Vessels of higher size were also positive.

The ESM-1 Gene in Humans and in Other Species—A Southern blot of genomic DNA from humans and other species (Zoo-blot, Clontech) was probed with a *Hind*III-digested ESM-1 fragment, which includes 1176 nucleotides at the 5' end, and washed at low stringency. Two major bands were revealed in human DNA with apparent sizes of 6.2 and 5.5 kb (Fig. 5). Two bands of similar intensity were also shown in monkey DNA. Their sizes were slightly greater than those in human DNA (6.7 and 6 kb). In addition, three bands at 8.2, 4, and 3.2 kb were seen in mouse; three bands at 5.5, 4, and 3.4 kb were seen in dog; and one band each, at 5.1, 2.7, and 2.4 kb, was also seen in

rat, cow, and rabbit, respectively. By contrast, no band was detected in chicken and yeast DNA. These data suggest that a single gene encodes ESM-1 and indicate the existence of a highly conserved ESM-1 gene between humans and primates and a related gene in other mammalian species.

ESM-1 Protein Expression—A 14-kDa COOH-terminal part of the ESM-1 protein was expressed as a fusion protein to glutathione *S*-transferase in *E. coli* (Fig. 6A). This protein was further purified as described under "Materials and Methods" and analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 6B). A polyclonal rabbit immune serum, raised against this product, was first assayed by immunoblotting. A 20-kDa protein was detected only in the supernatants from Sf9 cells infected by ESM-1 recombinant baculovirus (Fig. 6C, lane 4), consistent with the presence of a 20-kDa protein in these cell supernatants metabolically labeled with [35 S]methionine (Fig. 6C, lane 2). This was confirmed by the immunoprecipitation studies (data not shown). To localize the ESM-1 protein in COS cells, ESM-1 and mock transfectants were metabolically labeled. A protein with an apparent molecular mass of 20 kDa was immunoprecipitated only in supernatants from COS cells expressing ESM-1 (Fig. 6D, lane 2). A similar band was also found in COS cell lysates transfected with ESM-1. Absence of reducing agents in SDS-PAGE sample buffer had no effects on the mobility of ESM-1 in SDS-PAGE; under both conditions, ESM-1 migrated at 20 kDa (not shown), indicating that there are no disulfide-linked ESM-1 oligomers. To identify the presence of ESM-1 in HUVECs, we have performed Western blots of HUVEC lysates. A 20-kDa band was revealed with the anti-ESM-1 rabbit immune serum (Fig. 7B). This band appeared to be specific to HUVECs because no such band was seen in several other human cell line lysates including MO7, Daudi, HL60, and Jurkat. This 20-kDa band was further immunoprecipitated in supernatants and lysates from metabolically labeled HUVECs, giving similar results as ESM-1-transfected COS cell immunoprecipitation (Fig. 7A). Thus, the identical sizes of ESM-1 in HUVECs and in COS cells transfected with ESM-1 strongly indicate that the coding region of the ESM-1 cDNA is full length and the reading frame is correct.

Modulation of ESM-1 Expression by Cytokines—To determine the action of cytokines on ESM-1 expression, HUVECs were first treated with TNF α , and ESM-1 mRNA levels were examined by Northern blot. An up-regulation of ESM-1 mRNA was observed; it was time-dependent, peaking at 18 h after addition of TNF α (Fig. 8). The magnitude of this up-regulation was 3–4-fold the level of untreated cells and was constantly observed in five separate experiments. An early and transient down-regulation of ESM-1 mRNA could also be observed in three out of the five experiments, starting at 15 min and persisting up to 1 h 30 min after addition of TNF α (Fig. 8). To control the efficiency of the cytokine activation, these blots were reprobed with an E-selectin-specific DNA probe. A single 3.9-kb band corresponding to the E-selectin mRNA was shown to be rapidly and transiently up-regulated, peaking at the third hour after addition of TNF α (Fig. 8). Ethidium bromide gel staining and β -actin probing as controls confirmed that equal amounts of total RNA were present in each lane. HUVECs were also treated with IL-1 β , IL-4, and IFN γ . A pattern of regulation similar to that obtained with TNF α was seen with IL-1 β (data not shown). By contrast, no such change in ESM-1 mRNA level was observed with IL-4 and IFN γ , despite a time-dependent up-regulation of vascular cell adhesion molecule-1 (not shown) and ICAM-1 mRNA levels (Fig. 9). Unexpectedly, when combined with TNF α , IFN γ , but not IL-4, clearly inhibited TNF α -induced ESM-1 mRNA levels (Fig. 9). By contrast, IFN γ and TNF α displayed a synergistic induction of ICAM-1 gene expression. These data suggest that the expression

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Cloning and Expression of ESM-1

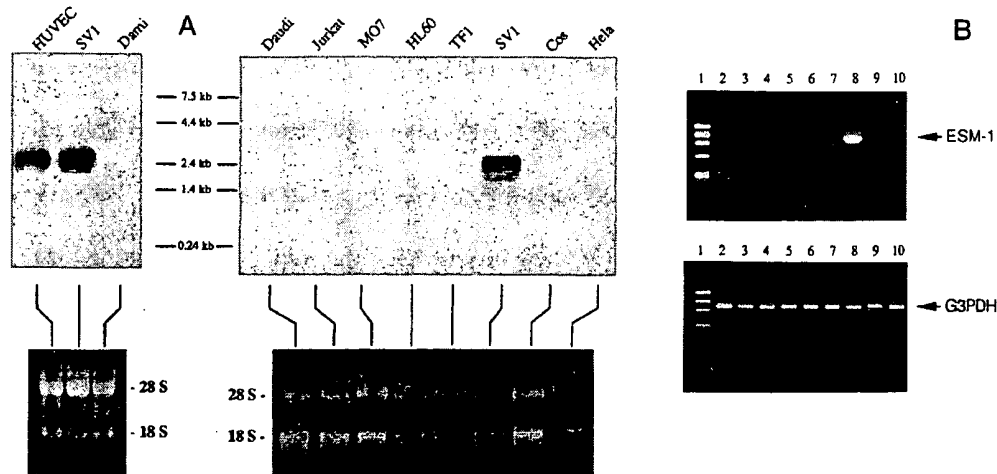


FIG. 3. Cellular distribution of ESM-1. A, multiple-cell Northern blot. 30 μ g of total RNA were applied per lane. The Northern blots were sequentially hybridized with two ESM-1 probes, including the whole cDNA sequence of ESM-1. The blots were washed in high stringency. The membranes were x-ray exposed for 1–2 weeks. The figure represents an autoradiogram exposed for 3 days. B, RT-PCR detection of ESM-1 transcripts in various human cell lines. The single strand cDNA was prepared from 5 μ g of total RNA of each cell type. The PCR was performed as described under "Materials and Methods." Lane 1, 1.6 μ g FX174 replicative form DNA/*Hae*III fragments (Life Technologies, Inc.); lane 2, Dami; lane 3, Daudi; lane 4, Jurkat; lane 5, MO7; lane 6, HL60; lane 7, TF1; lane 8, HUVEC; lane 9, COS; lane 10, HeLa.

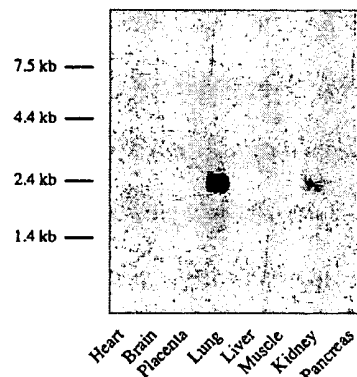


FIG. 4. Tissue distribution of ESM-1. Multiple human tissue Northern blot was purchased from Clontech. The membrane was washed in high stringency and x-ray exposed for 1 day to 2 weeks. The figure represents an autoradiogram exposed for 1 week.

of the ESM-1 gene is not only constitutive, but also is under the control of several cytokines.

DISCUSSION

In this study, we describe the isolation and molecular characterization of a novel human endothelial cell molecule designated ESM-1. This molecule displays several unique features of interest, which can confer on it a putatively important role in local inflammation.

Originally, the immunoscreening of a HUVEC cDNA library was designated to isolate a cDNA encoding a 55-kDa autoantigen putatively involved in severe asthma (13), and ESM-1 cDNA was further derived from one of the initially isolated cDNAs. It is unlikely that ESM-1 and the 55-kDa autoantigen are the same entity because: 1) the molecular sizes are quite different; 2) whatever the presence or the absence of reducing agents, the apparent molecular sizes of the 55-kDa autoantigen and ESM-1 did not change, arguing against the fact that the 55-kDa autoantigen may be an oligomer containing ESM-1; 3) more important, a Western blot of HUVEC lysates probed with the anti-ESM-1 antibodies did not reveal a 55-kDa band, and

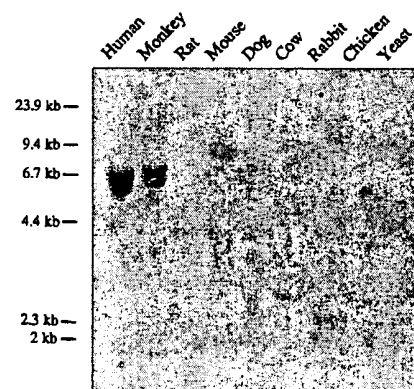


FIG. 5. Southern blot analysis of ESM-1 gene. The Zoo-blot (Clontech) was hybridized with the *Hind*III fragment of ESM-1 cDNA including the coding sequence of ESM-1. The membrane was washed two times for 30 min at room temperature in $2 \times$ SSC, 0.1% SDS, and two times for 30 min at 65 $^{\circ}$ C in the same buffer. The membrane was x-ray exposed for 4 days to 2 weeks. The figure represents the autoradiogram exposed for 2 weeks.

inversely, when probed with the anti-55-kDa antibodies, no band of 20 kDa was detected; and 4) although the 55-kDa autoantigen is also present in human megakaryoblastic cell lines such as Dami cells, ESM-1 mRNA could not be detected in this cell type by either Northern blot or RT-PCR. Thus, ESM-1 is clearly different from the 55-kDa autoantigen initially identified.

Sequence analysis confirmed a unique identity of ESM-1. Composition of the ESM-1 cDNA displays a large 3'-UTR containing a series of AU-rich elements (AREs). AU-rich elements are found in the 3'-UTR of many cytokine and oncogene mRNAs. The observation that AREs are involved in the mRNA destabilization (19, 20, 23, 24) further indicates that the mRNA for ESM-1 might be unstable. The 3'-UTR also contains five putative polyadenylation sites. Among them, only the last one appeared functional. Bands less than 2.2 kb were not found in HUVECs, either in the absence or in the presence of cytokines. Indeed, the use of *Hind*III-*Xba*I ESM-1 DNA fragment as a

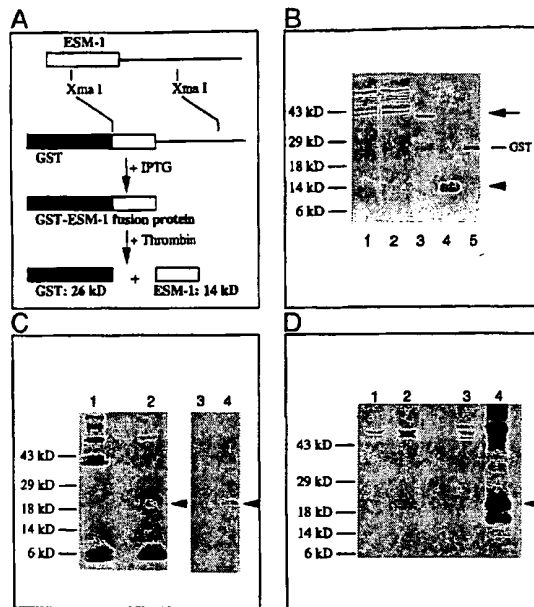


FIG. 6. ESM-1 protein expression. In *B*, *C*, *D*, arrowheads indicate ESM-1 protein. *A*, construction, production, and purification scheme of the 14-kDa COOH-terminal ESM-1 polypeptide. The *Xma*I-digested fragment of ESM-1 was ligated into the *Xma*I-digested and calf intestine alkaline phosphatase-treated pGEX-2TK vector. Detailed procedures are described under "Materials and Methods." *B*, SDS-PAGE analysis of ESM-1 protein product. Here is shown a 15% SDS-PAGE stained with Coomassie Brilliant Blue. Lanes 1 and 2, cleared bacterial lysate before (lane 1) and after (lane 2) passage through a glutathione-Sepharose 4B column; lane 3, glutathione *S*-transferase fusion protein eluted with 5 mM glutathione in Tris-HCl (pH 8); lane 4, purified COOH-terminal ESM-1 polypeptide cleaved by thrombin; lane 5, glutathione eluate from the column after thrombin treatment. Arrow indicates the presence of glutathione *S*-transferase-ESM-1 fusion protein. *C*, ESM-1 expression in Sf9 cells. Lanes 1 and 2, SDS-PAGE analysis of crude supernatants were run in 15% SDS-PAGE. Lane 1, Sf9 cells; lane 2, Sf9 cells infected with ESM-1 recombinant baculovirus; lanes 3 and 4, Western blotting of Sf9 cell supernatants expressing ESM-1. Supernatants from Sf9 were 10 × concentrated and 10 μl of each were run in 15% SDS-PAGE and transferred onto an Immobilon membrane (Amersham Corp.). The blots were probed with rabbit antiserum against ESM-1. Lane 3, Sf9 cells infected with hIL-5 recombinant baculovirus; lane 4, Sf9 cells expressing ESM-1. *D*, ESM-1 expression in COS cells. ESM-1 and mock transfectants were metabolically labeled for 6 h at the second day after transfection. COS cell supernatants and COS cell lysates were incubated first with 5 μl of rabbit antiserum and second with protein A-Sepharose CL-4B. After extensive washing, the beads were boiled in Laemmli buffer containing β-mercaptoethanol. Twenty microliters were run in 15% SDS-PAGE, dried, and autoradiographed. Immunoprecipitates from COS cell supernatants are shown in lane 1 (mock transfectant) and in lane 2 (ESM-1 transfectant). Those from COS cell lysates are shown in lane 3 (mock transfectant) and in lane 4 (ESM-1 transfectant).

probe (in which the greatest part of the 3'-UTR was deleted) did not reveal any band other than the 2.2-kb. This suggests that in our experimental conditions, only the last polyadenylation site was functional.

ESM-1 contains a hydrophobic NH₂-terminal amino acid sequence of 19 residues consistent with a signal sequence. This was confirmed both *in vitro* by the cleavage of the signal sequence in the presence of microsomal vesicles and *in vivo* by the presence of ESM-1 in supernatants of Sf9, COS cells expressing ESM-1, and finally in HUVECs. In addition, there is no transmembrane region, suggesting that ESM-1 is a secretory molecule. The presence of ESM-1 in cell supernatants were confirmed by immunoprecipitation in Sf9 cells, COS cells, and

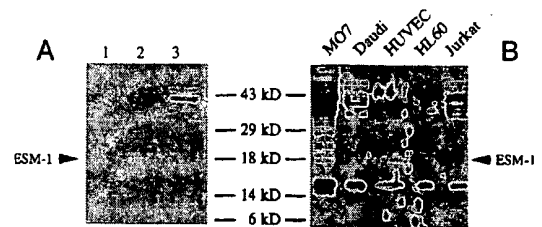


FIG. 7. Detection of the ESM-1 protein in HUVECs. *A*, immunoprecipitation of [³⁵S]methionine-labeled HUVECs. HUVECs (10⁶) were overnight labeled with [³⁵S]methionine. The cell supernatant and the cell lysate were precleared with 5 μl of control rabbit serum and 50 μl of protein A-Sepharose for 2 h at 4 °C. The cleared cell supernatant and cell lysate were then processed for immunoprecipitation as described for COS cells. Lane 1, HUVEC supernatant alone; lane 2, HUVEC supernatant incubated with 5 μl of rabbit anti-ESM-1 antiserum; lane 3, HUVEC lysate incubated with 5 μl of rabbit anti-ESM-1 antiserum. *B*, Western blot analysis of HUVEC lysate. Ten microliters of various cell lysates (containing 10⁶ cells) were analyzed in Western blot. The membrane was probed for 1 h with rabbit anti-ESM-1 antiserum (1:200 (v/v)) in PBS containing 0.1% Tween 20. After several washings, bound antibodies were labeled by a peroxidase-conjugated goat antirabbit immunoglobulin (1 h incubation at 1:2000 (v/v)) and revealed by ECL (Amersham Corp.).

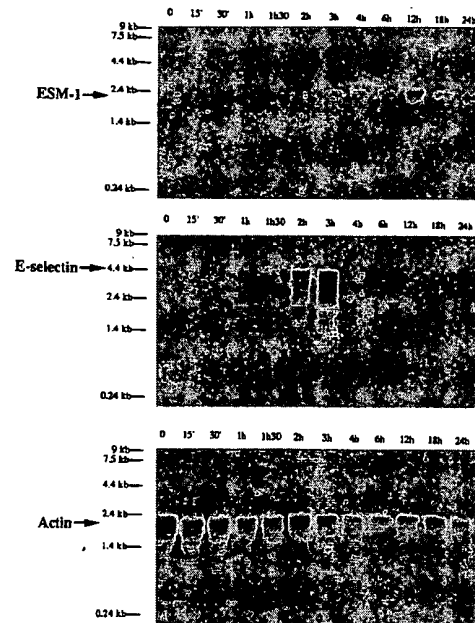


FIG. 8. Kinetic of TNFα-dependent ESM-1 gene up-regulation. HUVECs were cultured as described. TNFα (200 units/ml) was added to the culture medium. At different times the medium was removed and the cells were lysed in 4 M guanidine thiocyanate buffer. Total RNA was purified and separated in 1% formaldehyde agarose gel. A mean of 5–10 μg was extracted per 10⁶ HUVECs. The membranes were hybridized first with an ESM-1 probe (*Hind*III DNA fragment), second with an E-selectin probe (*Pst*I-*Eco*RI DNA fragment), and third with a β-actin probe (purchased from Clontech). The ESM-1 autoradiogram represents a 3-day x-ray exposure. The E-selectin and β-actin autoradiograms represent an overnight x-ray exposure.

HUVECs. The ESM-1 cDNA sequence predicts a mature ESM-1 polypeptide of 165 amino acids corresponding to a molecular mass of 20 kDa. This is in agreement with the *in vitro* translation assay and the immunoprecipitation from COS cells. In addition, immunoprecipitation and Western blot of HUVEC lysates confirmed the presence of ESM-1 at an identical apparent molecular mass of 20 kDa. These results indicate that 1) the ESM-1 cDNA is full length; 2) the correct reading frame is

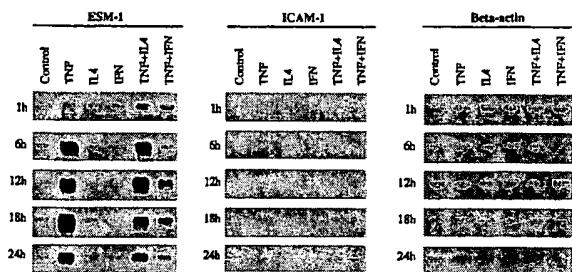


FIG. 9. *ESM-1* gene regulation by cytokines. Northern blot analysis of HUVECs stimulated with $\text{TNF}\alpha$, IL-4, IFN γ , $\text{TNF}\alpha$ + IL-4, and $\text{TNF}\alpha$ + IFN γ . HUVECs were cultured as described. Cytokines were added as indicated and RNA were recovered at 1, 6, 12, 18, and 24 h. Total RNA was electrophoresed and transferred as described. The membranes were hybridized first with a *ESM-1* probe (*Hind*III DNA fragment), second with an intercellular adhesion molecule 1 (ICAM-1) probe (*Eco*RI-*Xho*I DNA fragment), and third with a β -actin probe (purchased from Clontech).

synthesized; and 3) *ESM-1* is poorly glycosylated or unglycosylated both in COS cells and in HUVECs.

ESM-1 is a cysteine-rich molecule (10.9% cysteine residues). These cysteine residues are concentrated in the NH_2 -terminal part of the *ESM-1* molecule (110 amino acids). Because there are no disulfide links between monomers of *ESM-1*, these cysteines must be involved in intrachain disulfide bonds, which would confer to *ESM-1* a rigid structure. Following the cysteine-rich region of *ESM-1*, there is a COOH-terminal polypeptidic tail of 55 amino acids, which is free of cysteine residues, thereby indicating the presence of two distinct domains: a first domain of 110 amino acids and a second domain of 55 amino acids, characterized by the presence or the absence of cysteine residues, respectively.

An important finding is that the *ESM-1* mRNA is regulated by the cytokines. $\text{TNF}\alpha$ induced accumulation of *ESM-1* mRNA early; it was detectable at the second hour and peaked at the 18th hour of TNF incubation. In addition, IL-1 β was also shown to increase *ESM-1* gene expression in a very similar pattern as did $\text{TNF}\alpha$. Another interesting finding is the particular action of IFN γ on *ESM-1* mRNA expression. Usually, the addition of $\text{TNF}\alpha$ plus IFN γ to endothelial cells has a synergistic effect on the induction of expression of various proinflammatory factors. This is true for expression of class I major histocompatibility complexes (25), adhesion molecules (26), IL-6 (12), IL-8 (27), and RANTES (5). Unexpectedly, in the case of *ESM-1*, our results clearly indicate that IFN γ , which was without any effect when used alone, has inhibitory effects on $\text{TNF}\alpha$ -induced *ESM-1* gene expression. This suggests that *ESM-1* may exhibit unusual functions during the inflammatory reaction depending on cytokines.

A second finding is that this molecule is highly restricted to the endothelial cell, and that the *ESM-1* is synthesized, distributed, and restricted to the lung vascular endothelial cells. This would suggest that *ESM-1* may participate in specialized endothelial functions, particularly in the lung vascular spaces. In addition, *ESM-1* mRNA is also detected with less intensity in kidney, thereby indicating that vascular endothelium from lung and kidney may have common functional entities mediated by *ESM-1*. It is unclear why other endothelial cell-rich tissues, such as heart or placenta, have shown poor expression of *ESM-1* mRNA. One can suggest that the constitutive expres-

sion of *ESM-1* mRNA in the vascular endothelial cells may vary considerably, depending on either an organ-specific differentiation state of endothelial cells or specific factors present in the local environment. However, this point has to be confirmed by further immunohistological studies. It is also intriguing that the best fits found by computerized data base searches were amino acid sequences belonging to molecules involved in cell-cell or cell-matrix adhesion. The fact that the main cell types found in the airways is the monocyte-macrophage cell type would indicate that *ESM-1* may participate in the homing of this cell type from the blood stream to the alveolar spaces. On the other hand, taking into account the fact that endothelial cells exhibit constitutive expression of adhesion molecules, such as ICAM-1 and ICAM-2, at low level (6, 7), *ESM-1* may contribute to the inhibition of either the spontaneous leukocyte adhesion to the endothelium or the random migration of blood leukocytes through the vascular wall.

Taken collectively, these data would indicate a link between *ESM-1* and inflammation. Further purification of *ESM-1* and the production of monoclonal antibodies may provide the tools to analyze the exact biological role of *ESM-1*. This may result in new insights in the areas of vascular cell biology and human lung physiology.

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